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STEREOSPECIFIC REDUCTION OF PROGESTERONE BY *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; steroid metabolism; progesterone; 5α-pregnane-3,20-dione; 20β-hydroxy-4-pregnen-3-one; 5α-pregnane-3α,20β-diol.

Abstract—[4-^{14}C]-Progesterone was applied to the leaves of growing pea plants, *Pisum sativum*. After 3 weeks, about 50% of the administered steroid was reduced, about 20% being reduced to 5α-pregnane-3α,20β-diol as the major metabolite. The radioactivities of 5α-pregnane-3α,20α-diol and 5α-pregnane-3α,20β-diol after 3 weeks were more than twice those after one week. The following radioactive metabolites were also isolated: 5α-pregnane-3,20-dione; 20α-hydroxy-4-pregnen-3-one; 20β-hydroxy-4-pregnen-3-one; 3α-hydroxy-5α-pregnan-20-one; 3α-hydroxy-5β-pregnan-20-one; 3β-hydroxy-5α-pregnan-20-one; 20β-hydroxy-5α-pregnan-3-one; 5α-pregnane-3β,20β-diol; and 5β-pregnane-3α,20β-diol. The radioactivities of the 5α-pregnane derivatives were considerably higher than those of the corresponding 5β-pregnane derivatives.

INTRODUCTION

Progesterone has been isolated from higher plants, *Holarrhena floribunda* leaves and apple seeds [1]. We have previously demonstrated that plants, like animals, synthesize progesterone from pregnenolone [2, 3], which is a degradation product of cholesterol [4] or sitosterol [5]. Apparently, many plants have the ability to synthesize and metabolize progesterone [6]. Most of our knowledge about the metabolism of progesterone in plants comes from experiments with tissue homogenates, tissue cultures, and microorganisms.

Administration of labeled progesterone to growing plants has shown that *Digitalis lanata* [7] and *Strophanthus kombé* [8] reduce this steroid at C-3, C-5, and C-20. We have recently reported that *Pisum sativum* specifically reduces the administered 4-androstene-3,17-dione to testosterone [9]. Since androstenedione is derived from progesterone in animals [10], we were interested in studying progesterone metabolism in pea plants. One and three weeks after the foliar administration of radioactive progesterone, the radioactive progesterone metabolites were identified with the aid of high-pressure liquid chromatography (HPLC). Available TLC techniques [11] fail to resolve the complex mixture of progesterone reduction products, which are isomeric at C-3, C-5, and C-20. We have therefore devised methods for the separation of the reduction products of progesterone based on adsorption and reversed-phase partition HPLC [12]. These two HPLC systems are complementary, and by combining them we were able to isolate a broad spectrum of stereoisomers of pregnane derivatives in sufficiently pure form to permit crystallization to constant specific activity. We have thus identified eleven reduction products of progesterone.

RESULTS

[4-^{14}C]-Progesterone (10 μCi) was applied to the leaves of 2-week-old *P. sativum*. After 7 or 21 days, the whole plants were homogenized and boiled in acid. The neutral lipid fraction contained 70% and the acidic lipid fraction contained 3% of the total radioactivity. No radioactivity could be attributed to estrone, estradiol, estriol, and epiestriol in the acidic lipid fraction by addition of carriers, followed by TLC and co-crystallization [9].

The neutral lipid fraction was combined with non-radioactive carriers and the mixture was chromatographed by the reversed-phase HPLC system detailed in the legend of Fig. 1 [12]. Radioactive fractions from this chromatogram were then chromatographed by the adsorption HPLC system detailed in the legend of Fig. 2 [12]. The radioactive fractions from the second chromatogram were co-crystallized with appropriate reference steroids to constant specific radioactivities, as shown in Table 1. With this isotope dilution method we have examined a portion of the neutral lipid extract from *P. sativum* for the presence of the following possible progesterone metabolites: 20α-hydroxy-4-pregnen-3-one; 20β-hydroxy-4-pregnen-3-one; 5-pregnene-3β,20α-diol; 5-pregnene-3β,20β-diol; 5α-pregnane-3α,20α-diol; 5α-pregnane-3α,20β-diol; 5α-pregnane-3β,20α-diol; 5α-pregnane-3β,20β-diol; 5β-pregnane-3α, 20α-diol; 5β-pregnane-3α,20β-diol; 5β-pregnane-3β,20α-diol; 5β-pregnane-3β,20β-diol; 4-androstene-3,17-dione: testosterone; 5α-androstane-3β,17β-diol; and deoxycorticosterone (21-hydroxyprogesterone). The only radioactive metabolites of progesterone which we have detected are shown in Table 1. Side chain cleavage at C-17 and hydroxylation at C-21 of progesterone were not observed.

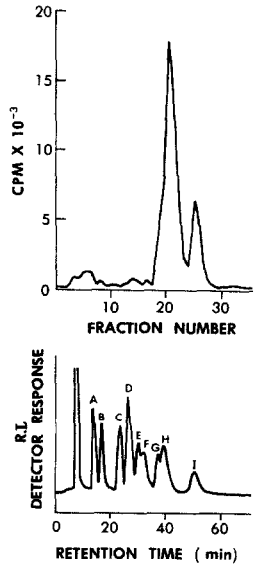


Fig. 1. Radiochromatogram of the neutral lipid fraction of *P. sativum*, one week after the administration of [4-^{14}C]-progesterone. Sample—2% of the neutral lipid fraction combined with 100 μg of each carrier in 100 μl of acetonitrile. Carriers—(A) testosterone; (B) 4-androstene-3,17-dione; (C) 5-pregnene-3β,20α-diol; (D) 20α-hydroxy-4-pregnen-3-one; (E) 5-pregnene-3β,20β-diol; (F) 5α-pregnane-3β,20α-diol; (G) 20β-hydroxy-4-pregnen-3-one; (H) 5α-pregnane-3β,20β-diol; (I) 5α-pregnane-3α,20β-diol. Column—Zorbax BP-ODS; 500 × 4 mm i.d.; eluant, 60% aqueous acetonitrile; flow rate, 1 ml/min: fraction, 2 ml; pressure, 1540 psi. RI detector—sensitivity 8 ×; recorder speed, 6 cm/hr; span, 10 mV.

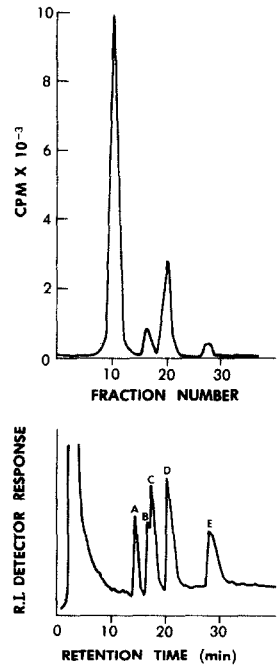


Fig. 2. Radiochromatogram of fractions G and H from Fig. 1. Sample, combined with 100 μg of each carrier in 100 μl of dichloromethane. Carriers—(A) 5β-pregnane-3β,20β-diol; (B) 5α-pregnane-3β,20β-diol; (C) 5α-pregnane-3α,20α-diol; (D) 20β-hydroxy-4-pregnen-3-one; (E) 5β-pregnane-3α,20β-diol. Column—Partisil 5, 600 × 2 mm i.d.; eluant, 0.25% ethanol in dichloromethane; flow rate, 1 ml/min; fraction, 1 ml; pressure, 3200 psi. RI detector—sensitivity 8 ×; recorder speed, 12 cm/hr; span, 10 mV.

Table 1. Recrystallization of steroids to constant specific radioactivity

	Compound	Solvent used for crystallization	cpm/μmol ±0.9 error
A	5α-Pregnane-3,20-dione	Methanol–water	47.9 ± 2.5
		Dichloromethane–hexane	44.5 ± 2.2
		Acetone–hexane	46.3 ± 2.3
B	20α-Hydroxy-4-pregnen-3-one	Methanol–water	36.5 ± 1.2
		Dichloromethane–hexane	35.5 ± 1.5
		Acetone–hexane	32.3 ± 1.6
	20α-Hydroxy-4-pregnen-3-one acetate	Methanol–water	33.1 ± 2.6
		Acetone–water	31.2 ± 2.5
		Acetonitrile–water	32.3 ± 2.2
C	20β-Hydroxy-4-pregnen-3-one	Methanol–water	240.9 ± 7.8
		Dichloromethane–hexane	249.6 ± 4.2
		Acetone–water	247.3 ± 7.4
	20β-Hydroxy-4-pregnen-3-one acetate	Methanol–water	243.9 ± 9.5
		Acetone–water	250.0 ± 8.9
		Acetonitrile–water	255.3 ± 10.6
D	3α-Hydroxy-5α-pregnan-20-one	Methanol–water	29.1 ± 2.2
		Dichloromethane–hexane	26.2 ± 1.9
		Acetone–water	27.1 ± 2.6
	3β-Hydroxy-5α-pregnan-20-one acetate	Methanol–water	26.6 ± 1.4
		Acetone–water	29.1 ± 2.0

Table 1. (*continued*)

	Compound	Solvent used for crystallization	cpm/μmol ±0.9 error
		Acetonitrile–water	30.5 ± 2.2
E	3α-Hydroxy-5β-pregnan-20-one	Methanol–water	17.6 ± 1.7
		Dichloromethane–hexane	16.8 ± 1.6
		Acetone–water	17.6 ± 1.6
	3α-Hydroxy-5β-pregnan-20-one acetate	Methanol–water	18.0 ± 1.8
		Acetone–water	17.2 ± 1.6
		Acetonitrile–water	17.6 ± 1.7
F	3β-Hydroxy-5α-pregnan-20-one	Dichloromethane–hexane	124.4 ± 3.8
		Acetone–water	122.6 ± 4.1
		Methanol–water	123.4 ± 3.6
	3β-Hydroxy-5α-pregnan-20-one acetate	Methanol–water	118.7 ± 4.2
		Acetone–water	122.1 ± 5.2
		Acetonitrile–water	123.4 ± 4.8
G	20β-Hydroxy-5α-pregnan-3-one	Methanol–water	23.0 ± 2.2
		Dichloromethane–hexane	21.6 ± 1.9
		Acetone–water	23.0 ± 2.1
	20β-Hydroxy-5α-pregnan-3-one acetate	Methanol–water	23.6 ± 1.8
		Acetone–water	23.2 ± 1.6
		Acetonitrile–water	23.0 ± 1.9
H	5α-Pregnane-3α,20α-diol	Methanol–water	23.2 ± 2.3
		Tetrahydrofuran–water	25.7 ± 2.3
		Acetone–water	23.6 ± 2.5
	5α-Pregnane-3α,20α-diol diacetate	Acetone–water	22.7 ± 2.2
		Methanol–water	27.6 ± 2.2
		Acetonitrile–water	22.1 ± 2.4
I	5α-Pregnane-3α,20β-diol	Methanol–water	768.0 ± 9.6
		Acetone–water	776.9 ± 12.7
		Tetrahydrofuran–water	768.3 ± 11.4
	5α-Pregnane-3α,20β-diol diacetate	Methanol–water	798.8 ± 8.8
		Acetone–water	795.0 ± 13.9
		Acetonitrile–water	769.6 ± 15.1
J	5α-Pregnane-3β,20β-diol	Methanol–water	46.9 ± 2.6
		Acetone	43.4 ± 2.5
		Tetrahydrofuran–hexane	45.1 ± 2.5
	5α-Pregnane-3β,20β-diol diacetate	Methanol–water	45.0 ± 2.8
		Acetone–water	45.0 ± 2.4
		Acetonitrile–water	45.8 ± 2.8
K	5β-Pregnane-3α,20β-diol	Methanol–water	24.7 ± 1.3
		Acetone	23.1 ± 1.3
		Tetrahydrofuran–hexane	24.0 ± 1.4
	5β-Pregnane-3α,20β-diol diacetate	Methanol–water	23.4 ± 1.8
		Acetone–water	23.3 ± 1.7
		Acetonitrile–water	23.7 ± 1.7

Another portion of the neutral lipid fraction was then combined with the following non-radioactive carriers: 4-pregnene-3,20-dione (progesterone); 5-pregnene-3,20-dione; 5α-pregnane-3,20-dione; 5β-pregnane-3,20-dione; 3β-hydroxy-5-pregnen-20-one (pregnenolone); 3α-hydroxy-5α-pregnan-20-one; 3α-hydroxy-5β-pregnan-20-one; 3β-hydroxy-5α-pregnan-20-one; 3β-hydroxy-5β-pregnan-20-one; 20α-hydroxy-5α-pregnan-3-one; 20α-hydroxy-5β-pregnan-3-one; 20β-hydroxy-5α-pregnan-3-one; and 20β-hydroxy-5β-pregnan-3-one. The mixture was chromatographed by adsorption HPLC (cf. Fig. 2) with the eluant 0.25% ethanol in dichloromethane [12]. The radioactive fractions thus obtained were then chromatographed by reversed-phase HPLC (cf. Fig. 1). The radioactive fractions from this chromatogram were co-crystallized with the appropriate reference steroids to constant specific radioactivities, as shown in Table 1. Table 2 shows the radioactivity incorporated into each of the metabolites 7 and 21 days after the application of radioactive progesterone in percent of the administered dose.

DISCUSSION

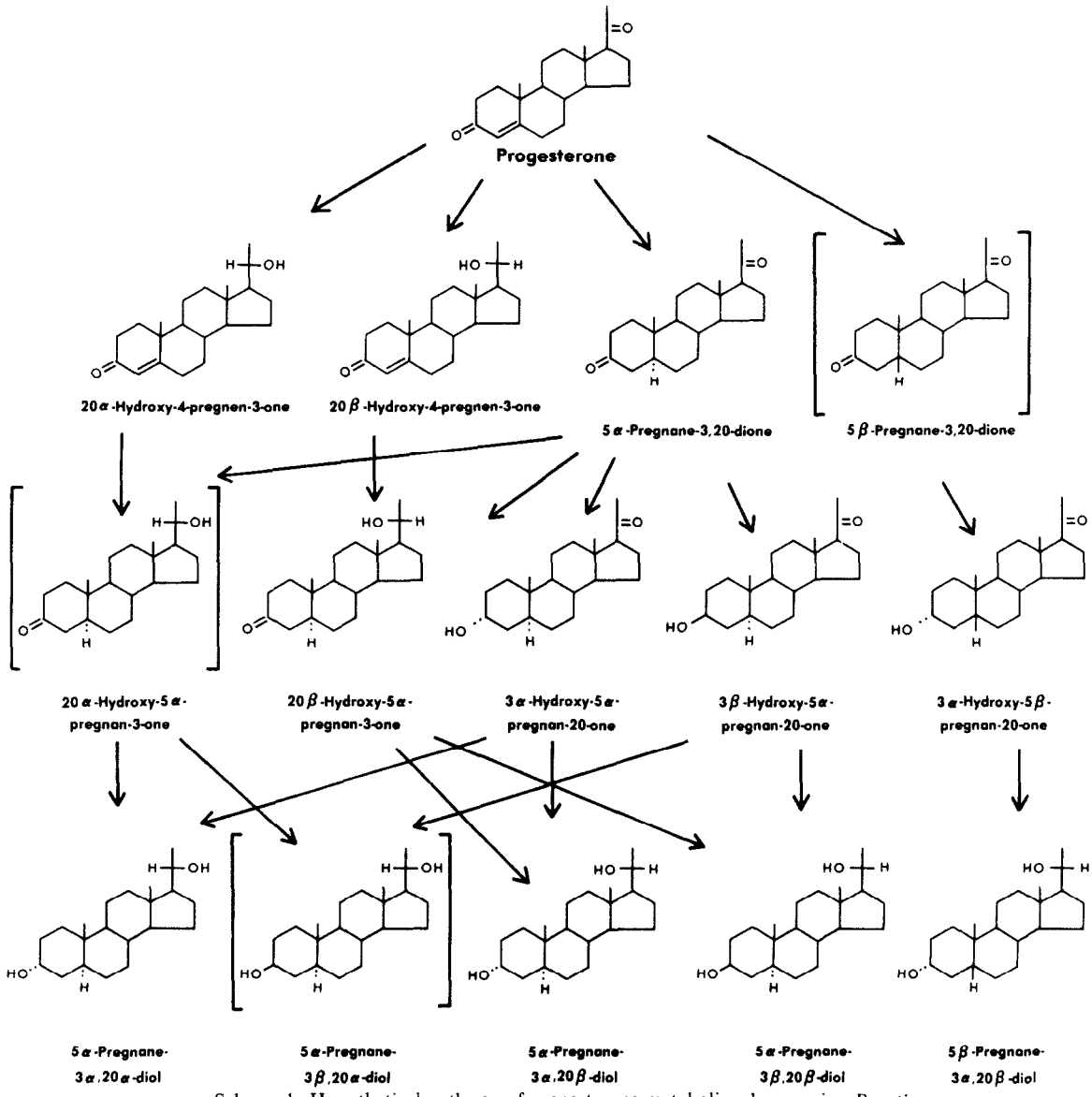
P. sativum has the ability to reduce administered progesterone to the products shown in Table 2. However, no conversion to 4-androstene-3,17-dione; testosterone; 5α-androstane-3β,17β-diol; estrone; estradiol; estriol;

Table 2. Metabolism of [4-^{14}C]-progesterone by growing *P. sativum*. Percent of administered radioactivity 1 and 3 weeks after the administration of progesterone

Compound	1 week	3 weeks
Progesterone	30.4	21.9
5α-Pregnane-3,20-dione	3.7	2.3
20α-Hydroxy-4-pregnen-3-one	1.7	1.3
20β-Hydroxy-4-pregnen-3-one	9.2	7.1
3α-Hydroxy-5α-pregnan-20-one	3.1	2.5
3α-Hydroxy-5β-pregnan-20-one	1.3	1.3
3β-Hydroxy-5α-pregnan-20-one	5.1	5.4
20β-Hydroxy-5α-pregnan-3-one	1.5	1.3
5α-Pregnane-3α,20α-diol	0.4	1.0
5α-Pregnane-3α,20β-diol	8.7	19.7
5α-Pregnane-3β,20β-diol	3.1	4.2
5β-Pregnane-3α,20β-diol	1.9	2.0

and epiestriol was observed. Although steroid hormones occur in higher plants [1], most of the information about their biosynthesis comes from work on microorganisms. Good evidence has been presented for the biosynthesis of estradiol from mevalonic acid and estrone in *Phaseolus vulgaris* [13] and for the biosynthesis of deoxycorticosterone from progesterone in *D. lanata* [14]. In *P. sativum*, reduction is the major route by which progesterone is metabolized, about half of the administered dose being reduced in 3 weeks. From the data in Table 2 it is evident that among the reduction products of progesterone the 5α-pregnane derivatives predominate over the 5β-pregnane derivatives. This agrees with our observations in *D. lanata* [7] and *S. kombé* [8].

Similarly, leaf homogenates of *Cheiranthus cheiri*, *D. purpurea*, *S. kombé*, and *Corchorus olitorius* preferentially metabolized progesterone to 5α-pregnane derivatives [15]. When such leaf homogenates were incubated with 3β-hydroxy-5α- and 3β-hydroxy-5β-pregnan-20-one, only the 5α-epimer was metabolized [16].



Scheme 1. Hypothetical pathway of progesterone metabolism by growing *P. sativum*.

Microsomes from *C. cheiri* and *Dioscorea deltoidea* metabolized progesterone exclusively to 5α-pregnane-3,20-dione [17]. *D. deltoidea* suspension cultures converted progesterone to 3β-hydroxy-5α-pregnan-20-one and 5α-pregnane-3β,20β-diol [18]. The conversion to 5β-pregnane-3,20-dione and to 3β-hydroxy-5α-pregnan-20-one was also observed in various other plant tissue cultures [19, 20], and *D. purpurea* suspension cultures, likewise, gave conversion products of the 5α-pregnane series exclusively [21].

As shown in Table 2, there is also a predominance in the incorporation of radioactivity into the 20β-pregnane derivatives. The most highly radioactive metabolite, 5α-pregnane-3α,20β-diol, is 20 times more radioactive than its 20α-epimer. Similarly, 20β-hydroxy-4-pregnen-3-one is more radioactive than its 20α-epimer and, while 20β-hydroxy-5α-pregnane-3-one showed some radioactivity, none could be detected in its 20α-epimer.

Our results are summarized in Scheme 1, which shows a hypothetical pathway of progesterone metabolism in *P. sativum*. Intermediates postulated, but not actually isolated, are shown in brackets. In the first step, reduction takes place at C-5 or at C-20. These reactions show a stereospecific bias in favor of 5α- and 20β-reduction. While the radioactivity in progesterone, naturally, decreases between the first and third week after administration, the radioactivities of these intermediates remain unchanged (cf. Table 2), indicating an equilibrium between their formation and transformation.

In the second step, the 3-ketones and 20-ketones resulting from the first step are reduced to monohydroxy-monoketones, and ring A is saturated, again with a stereospecific bias. Again, the radioactivity remains unchanged (cf. Table 2), the intermediates being transformed at the approximate rate of formation. Finally, in the third step, reduction is complete and products accumulate, as is evident from their rising radioactivity (cf. Table 2). However, 5β-pregnene-3α,20β-diol appears to be in a steady state, indicating that it is undergoing further metabolism.

In pea plants 5α-pregnane-3α,20β-diol appears to be an end-product of progesterone metabolism. In contrast, in man and dog the chief urinary metabolite of progesterone, pregnanediol, is the 5β-pregnane-3α,20α-diol [10].

EXPERIMENTAL

Methods. The HPLC apparatus was assembled from commercially available components [12]. About 100 μl of sample was injected into the sample injector (Model 7125, Rheodyne, Berkeley, California) with a Hamilton syringe. An event marker recorded each change of the fraction collector (Model 7000 Ultrorac, LKB, Rockville, Maryland) on a recorder (Model 385, Linear, Irvine, California) together with the UV detector (Model 155, Altex, Berkeley, California) response or RI detector (Model R-401, Waters, Milford, Massachusetts) response. An aliquot of each fraction was counted in a scintillation counter. The columns were packed in our laboratory with a slurry packer (Model 29426, Haskel, Burbank, California). For adsorption HPLC, Partisil 5 (5 μm, Whatman, Clifton, New Jersey) was packed into a column, 600 × 2 mm i.d., and for reversed-phase HPLC, Zorbax BP-ODS (7–8 μm, Dupont de Nemours, Wilmington, Delaware) was packed into a column, 500 × 4 mm i.d.

Administration of [4-^{14}C]-*progesterone.* The dwarf pea plants *P. sativum* var. Progress No. 9 were grown in a greenhouse. [4-^{14}C]-Progesterone (10 μCi, 51 mCi/mmol, New England Nuclear, Boston, Massachusetts) was dissolved in 25 μl of 95% EtOH, containing 0.1% DL-α-tocopherol and 0.1% of silicone oil DC-200 [22]. The soln was applied to the upper leaf surface of each plant when it was *ca* 2 weeks old.

Extraction of radioactive metabolites. Seven days or 21 days after the administration, entire plants were homogenized, and the homogenate was refluxed with HCl and extracted, as previously described [9]. Non-radioactive estorgen carriers, 1 mg each of estrone, estradiol, 16-epiestriol and estriol were added before hydrolysis. The estrogens in the acidic lipid fraction were analysed as previously described [9]. No radioactivity was associated with these estrogens.

Isolation and identification of radioactive metabolites. A 2% aliquot of neutral lipid fraction was combined with non-radioactive carriers and the mixture was chromatographed on the reversed-phase column (Fig. 1). Peak G + H in Fig. 1 contained mostly radioactive progesterone. This fraction was combined with non-radioactive carriers which were also eluted in this fraction. The mixture was chromatographed on an adsorption column (Fig. 2). Each of the fractions A–E in Fig. 2 was combined with 10 mg of the appropriate reference steroid. Crystallizations were repeated until the specific radioactivity was constant (Table 1). Since 5α-pregnane-3β,20β-diol and 5α-pregnane-3α,20α-diol could not be separated completely (Fig. 2), half of the combined fraction was used for each co-crystallization. The results showed that 5α-pregnane-3β,20β-diol, 5α-pregnane-3α,20α-diol, 20β-hydroxy-4-pregnen-3-one, and 5β-pregnane-3α,20β-diol were radioactive.

The fraction containing 5α-pregnane-3α,20β-diol (Peak I in Fig. 1) was chromatographed under the conditions shown in Fig. 2 to remove the radioactive progesterone and then recrystallized with carrier material. 5α-Pregnane-3α,20β-diol was found to be the most highly radioactive metabolite. The radioactive fraction between 20α-hydroxy-4-pregnen-3-one (D) and 5α-pregnane-3β,20α-diol (F) in Fig. 1 was combined with the non-radioactive carriers, 20α-hydroxy-4-pregnen-3-one, 5-pregnen-3β,20β-diol, 5β-pregnane-3β,20α-diol, 5α-pregnane-3β,20α-diol, and 5β-pregnane-3α,20α-diol. This mixture was then chromatographed under the conditions shown in Fig. 2. The only radioactive fraction detected was associated with 20α-hydroxy-4-pregnen-3-one. The fraction corresponding to testosterone (A) and androstenedione (B) in Fig. 1 was combined with the non-radioactive carriers, testosterone, deoxycorticosterone, androstenedione, and 5α-androstane-3β,17β-diol. This mixture was chromatographed under the conditions shown in Fig. 2 and was completely resolved. No radioactivity was associated with any of these carriers.

Another 2% of the neutral lipid fraction was combined with progesterone; 5-pregnene-3,20-dione; 5α-pregnane-3,20-dione; 5β-pregnane-3,20-dione; 3β-hydroxy-5-pregnen-20-one; 3α-hydroxy-5α-pregnan-20-one; 3α-hydroxy-5β-pregnan-20-one; 3β-hydroxy-5α-pregnan-20-one; 3β-hydroxy-5β-pregnan-20-one; 20α-hydroxy-5α-pregnan-3-one; 20α-hydroxy-5β-pregnan-3-one; 20β-hydroxy-5α-pregnan-3-one; and 20β-hydroxy-5β-pregnan-3-one. This mixture was chromatographed under the conditions shown in Fig. 2, except for the use of a UV detector at 280 nm and 0.25% EtOH in CH_2Cl_2 as the eluant. This chromatogram is published elsewhere [12]. The radioactive fractions from this chromatogram were rechromatographed under the conditions shown in Fig. 1. The radioactive fractions were then recrystallized with the carriers. The results showed that only 5α-pregnane-3,20-dione; 3α-hydroxy-5α-pregnan-20-one; 3α-hydroxy-5β-pregnan-20-one; 3β-hydroxy-5α-pregnan-20-one; and 20β-hydroxy-5α-pregnan-3-one were radioactive.

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